INHIBITOR-RESISTANT HCV NS3 PROTEASE

RELATED APPLICATIONS

Benefit of US Provisional Application, Serial No. 60/421,943, filed on October 29,
2002, is hereby claimed, and said application is herein incorporated by reference in
its entirety.

FIELD OF THE INVENTION

The present invention relates to a novel HCV NS3 protease, and in particular, to an
inhibitor-resistant HCV NS3 protease comprising a mutated amino acid sequence. It
also relates to methods of using such an inhibitor-resistant protease to identify
inhibitors having activity against HCV strains which have developed resistance to
known treatments.

BACKGROUND

Hepatitis C virus (HCV) is the major etiological agent of post-transfusion and
community-acquired non-A non-B hepatitis worldwide. It is estimated that over 200
million people worldwide are infected by the virus. A high percentage of carriers
become chronically infected and many progress to chronic liver disease, or so-called
chronic hepatitis C. This group is in turn at high risk for serious liver disease such as
liver cirrhosis, hepatocellular carcinoma and terminal liver disease leading to death.

The mechanism by which HCV establishes viral persistence and causes a high rate of
chronic liver disease has not been thoroughly elucidated. It is not known how HCV
interacts with and evades the host immune system. In addition, the roles of cellular
and humoral immune responses which protect against HCV infection and disease
have yet to be established.

HCV is an enveloped positive strand RNA virus of the *Flaviviridae* family. The
single strand HCV RNA genome is of positive polarity and comprises one open
reading frame (ORF) of approximately 9600 nucleotides in length, which encodes a

linear polyprotein of approximately 3010 amino acids. In infected cells, this polyprotein is cleaved at multiple sites by cellular and viral proteases to produce structural and non-structural (NS) proteins. The structural proteins (C, E1, E2 and E2-p7) comprise polypeptides that constitute the virus particle. The non-structural

5   proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) encode for enzymes or accessory factors that catalyze and regulate the replication of the HCV RNA genome. Processing of the structural proteins is catalyzed by host cell proteases. The generation of the mature non-structural proteins is catalyzed by two virally encoded proteases. The first is the NS2/3 zinc-dependent protease which auto-catalyzes the

10   release of the NS3 protein from the polyprotein. The released NS3 protein contains an N-terminal serine protease domain and catalyzes the remaining cleavages from the polyprotein. The released NS4A protein has at least two roles. The first role is to form a stable complex with NS3 protein and assist in the membrane localization of the NS3/NS4A complex. The second role of the NS4A protein is to act as a cofactor

15   for NS3 protease activity. This membrane-associated complex in turn catalyzes the cleavage of the remaining sites on the polyprotein, thus effecting the release of NS4B, NS5A and NS5B. The C-terminal segment of the NS3 protein also harbors nucleoside triphosphatase and RNA helicase activity. The function of the NS4B protein is unknown. NS5A is a highly phosphorylated protein that appears to be

20   responsible for the interferon resistance of various HCV genotypes. NS5B is an RNA-dependent RNA polymerase (RdRp) that is involved in the replication of HCV.

The open reading frame of the HCV RNA genome is flanked on its 5' end by a non-translated region (NTR) of approximately 340 nucleotides that functions as the internal ribosome entry site (IRES), and on its 3' end by an NTR of approximately

25   230 nucleotides. Both the 5' and 3' NTRs are important for RNA genome replication. The genomic sequence variance is not evenly distributed over the genome and the 5'NTR and parts of the 3'NTR are the most highly conserved portions.

The cloned and characterized partial and complete sequences of the HCV genome have been analyzed with regard to appropriate targets for prospective antiviral

30   therapies. The following four viral enzyme activities provide possible targets: (1) the NS2/3 protease; (2) the NS3/4A protease complex, (3) the NS3 helicase and (4) the

-2-

NS5B RNA-dependent RNA polymerase (NS5B RdRp). The NS3 protease has also been crystallized to reveal a structure reminiscent of other serine proteases (**Love** *et al.*, **1996; Kim** *et al.* **1996**).

5   NS3 protease activity is an attractive target for drug discovery. Enzymatic studies have shown that peptides based on the N-terminal product of the NS5A/5B cleavage site are competitive inhibitors of the enzyme. These peptides have served as a useful starting point in medicinal chemistry efforts to rationally design NS3 protease inhibitors as clinically effective anti-HCV compounds.

10   Chronic hepatitis C has emerged as an important clinical indication, an effective treatment for which has yet to be developed due to poor response rates to currently existing treatments. For example, the newly approved standard treatment, pegylated-interferon in combination with Ribavirin, exhibits a sustained response rate of 40 to 50%. However, a majority of patients still do not elicit a sustained anti-viral response, particularly against the interferon-resistant HCV genotypes, 1a and 1b.

15   WO 00/09543, WO 00/09558 and WO 00/59929 (all three incorporated herein by reference) disclose certain types of inhibitors of the HCV NS3 protease that are highly active and selective. These compounds have potential for becoming the next generation of anti-HCV treatment. It can be expected that these inhibitors, as well as many other antiviral treatments, will eventually give rise to viruses that are at least

20   partially resistant to such inhibitors.

Knowledge of mutations which render HCV resistant to inhibitors provides the basis for identifying inhibitors that are effective against such resistant strains. **Trozzi** *et al.*, **2003** has disclosed a resistant mutant replicon having three individual amino acid substitutions (D168A/Y/V) that render the protease resistant to an inhibitor of the

25   NS3 protease.

Accordingly, in an effort to develop a treatment with long-term efficacy that suppresses or overcomes anti-HCV resistance, we describe a means to identify anti-HCV compounds that exhibit activity against inhibitor resistant HCV strains.

SUMMARY OF THE INVENTION

In a first embodiment, the present invention provides a method for selecting a mutant inhibitor-resistant HCV NS3 protease comprising the steps of:

- preparing a nucleic acid construct encoding a selectable marker and native
  HCV NS3 protease and transfecting host cells with said construct; and

- incubating the transfected host cells in the presence of an HCV NS3 inhibitor under conditions suitable for selection of transfected cells, wherein colonies resulting from incubation under these conditions render mutant inhibitor-resistant HCV NS3 protease.

According to another aspect of this first embodiment, the present invention provides a method for isolating a mutant inhibitor-resistant HCV NS3 protease wherein the method described above further comprises the steps of:

- lysing the cells to release the RNA and using such RNA to produce the resistant-mutant protease; or lysing the cells to release the inhibitor-resistant NS3 protease; and isolating said inhibitor-resistant NS3 protease.

In this regard, the present invention provides in a second embodiment, novel inhibitor-resistant HCV NS3 proteases.

In this regard, the present invention provides in a second embodiment, novel isolated inhibitor-resistant HCV NS3 proteases.

In a third embodiment, the present invention provides recombinant nucleic acids encoding inhibitor-resistant HCV NS3 proteases.

Still, in a third embodiment, the present invention provides isolated recombinant nucleic acids encoding inhibitor-resistant HCV NS3 proteases.

According to another aspect of this third embodiment, there is provided a nucleotide probe capable of hybridizing under stringent conditions to a mutated nucleotide sequence as defined herein for diagnostic purposes.

In a fourth embodiment, vectors incorporating nucleic acids encoding inhibitor-resistant HCV NS3 proteases are provided.

-4-

Host cells transfected with these vectors, and cell lines derived therefrom, are provided in a fifth embodiment of the invention.

In a sixth embodiment, the invention encompasses a method for evaluating HCV NS3 protease activity of inhibitor-resistant NS3 proteases in accordance with the invention, comprising the steps of:

- incubating host cells transfected with nucleic acid encoding an inhibitor-resistant HCV NS3 protease under conditions which cause the protease to be expressed; and

- measuring the replication of the nucleic acid in the host cells, wherein the level of replication is proportional to the activity of the expressed protease.

In a seventh embodiment, the invention encompasses a method for identifying potential second generation inhibitors of HCV NS3 protease activity comprising:

- incubating host cells transfected with nucleic acid encoding an inhibitor-resistant NS3 protease under conditions which cause expression thereof, in the absence of a candidate second generation inhibitor compound;

- incubating host cells transfected with nucleic acid encoding an inhibitor-resistant NS3 protease under conditions which cause expression thereof, in the presence of a candidate second generation inhibitor compound; and

- measuring the replication of the nucleic acid in the presence and absence of the candidate second generation inhibitor compound, wherein the level of replication of the nucleic acid is proportional to the activity of the expressed protease, and wherein a decrease in activity of the protease in the presence of a candidate second generation inhibitor compound indicates that the compound inhibits the protease.

In an eighth embodiment, the invention encompasses a method for identifying potential second generation inhibitors of HCV NS3 protease activity comprising:

- incubating an inhibitor-resistant NS3 protease mutant as defined above in the presence or absence of a candidate second generation inhibitor compound; and

-5-

- measuring the protease activity of the inhibitor-resistant NS3 protease in the presence and absence of the candidate second generation inhibitor compound;

wherein a decrease in activity in the presence of a candidate second generation inhibitor compound indicates that the compound inhibits the inhibitor-resistant NS3 protease.

Other objects, advantages and features of the present invention will become more apparent upon reading the following non-restrictive description of the preferred embodiments with reference to the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the method by which HCV RNA replication was established in Huh-7 cell culture;

Figure 2 illustrates neomycin-resistant Huh-7 cell growth in the presence of NS3 protease inhibitor at concentrations ranging from 2 nM to 2000 nM; and

Figure 3 illustrates the clustering of several amino acid substitutions selected with the NS3 protease inhibitors to amino acids that are proximal to the active site (light grey) of the NS3 protease domain . The residues in white indicate the ones identified in resistance studies and located near the bound inhibitor in crystal structure (structure reported in Y.S. Tsantrizos, et al, Angew Chemie v42, 1356).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell culture, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook *et al*. (2000) and Ausubel *et al*. (1994).

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission (1972).

5   The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

The term "recombinant DNA", "recombinant nucleic acid molecule" or "recombinant plasmid" as known in the art refers to a DNA molecule resulting from the joining of

10   DNA segments. This is often referred to as genetic engineering.

The term "nucleic acid" as it is used with respect to segments, molecules or sequences refers to a unit comprised of nucleotides and, thus, encompasses both DNA molecules and RNA molecules. These segments, molecules or sequences can be isolated from natural sources using techniques established in the art, or can be

15   synthetically derived also using well-established techniques. When read in accordance with the genetic code, these sequences can encode a linear stretch or sequence of amino acids which define a polypeptide, a protein or a protein fragment.

The term "DNA" as it is used with respect to segments, molecules or sequences, is used herein to refer to a chain of nucleotides, each containing the sugar deoxyribose

20   and one of the four bases, adenine (A), guanine (G), thymine (T) or cytosine (C). The term "RNA" refers to a chain of nucleotides, each containing the sugar ribose and one of the four bases adenine (A), guanine (G), uracil (U) or cytosine (C). As one of skill in the art will appreciate, references to DNA which follow are also generally applicable to RNA.

25   The term "derivative" denotes a modification which comprises the addition of a chemical moiety that imparts on the NS3 mutant protease, or DNA encoding it, one or more desirable properties. Such chemical moieties, for example, can impart improved stability (i.e. biological half life). These moieties can also be used for the purpose of labeling. Moieties capable of providing these and other desirable

30   properties can be found in *Remington's The Science and Practice of Pharmacy*

-7-

(1995). Methodologies for coupling such moieties to a molecule are well known in the art.

The term "fragment" refers to a segment of an identified inhibitor-resistant mutant NS3 protease-encoding DNA, RNA or amino acid sequence, and/or a segment of any variant or derivative thereof, that substantially retains the capacity to encode an inhibitor-resistant NS3 protease. Such fragments may include, but are not limited to, 5' or 3' truncated nucleotide sequences or terminally truncated amino acid sequences.

The term "expression vector" defines a vector similar to that described above which additionally incorporates the elements necessary to enable the expression of an inserted sequence following transformation or transfection into a host. The cloned gene (inserted sequence) is usually placed under the control of expression control element sequences such as promoter sequences. Such expression control sequences will vary depending on whether the vector is designed to express the inserted sequence in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites. DNA/RNA is herein referred to as being incorporated "expressibly" into such a vector, and incorporated "expressibly" into a cell, once successful expression from a cell is achieved.

By "eukaryotic expression system" is meant the combination of an appropriate expression vector and a eukaryotic cell line which can be used to express a gene of interest. In all cases, the vector will contain appropriate control elements (promoter) to express the gene in the cell type of interest. Eukaryotic cell types typically used include yeast cells (e.g. *Saccharomyces cerevisiae, Pischia pastoris)* transfected with a plasmid vector; and mammalian cells transfected with DNA vectors for transient or constitutive expression. A preferred cell line useful for the purpose of this invention is derived from liver tissue.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence whether a nucleic acid or amino acid sequence, a

-8-

molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. In the present instance, the functional derivative means that the resulting amino acid sequence retains at least a portion of the biological activity of the NS3 protease sufficient to allow the NS3

5   protease to cleave at least a portion, preferably all its natural substrates i.e. the NS3/4A, 4A/4B, 4B/5A and 5A/5B cleavages sites. *In vivo*, the NS3 protease activity is retained when the HCV RNA or the virus is able to replicate. This functional derivative or equivalent may be a natural derivative or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or

10   additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid has chemico-physical

15   properties which usually, but not necessarily, are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophilicity and the like. Some of the most commonly known conservative amino acid substitutions include, but are not limited to:

Leu or Val or Ile; Gly or Ala; Asp or Glu; Asp or Asn or His; Glu or Gln; Lys or

20   Arg;

Phe or Trp or Tyr; Val or Ala; Cys or Ser; Thr or Ser; and Met or Leu.

The expression "hybridizing under stringent conditions" as used herein means conditions that distinguish at least one or more nucleotide change in the target sequence.

25   As used herein, the term "inhibitor-resistant" is meant to refer to a mutant HCV NS3 protease that substantially maintains protease activity in the presence of a compound generally known to inhibit native HCV NS3 protease activity. For clarity, "an inhibitor" is a compound that inhibits the cleavage of one or more of the NS3 protease cleavage sites selected from: NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B.

30   The term "isolated" as used herein means in a state isolated from the cell i.e. any cell-free extract.

For clarity, "mutant HCV NS3 protease" refers to HCV NS3 protease including one or more amino acid mutations, such as amino acid modifications, substitutions, deletions and insertions, that do not result in the elimination of protease activity. As will be understood by one of skill in the art, the activity of a mutant HCV NS3

5   protease, may vary somewhat from that of native HCV NS3 protease. It will be understood by those of skill in the art, the present invention is meant to encompass inhibitor-resistant mutant NS3 protease and is not, thus, limited to the specific amino acid sequences exemplified herein. The invention also encompasses derivatives or variants of the present mutant NS3 proteases, or fragments thereof, which exhibit

10   resistance to HCV NS3 protease inhibitors. As exemplified herein below, the nucleotide sequences and polypeptides used in the present invention can be modified, for example by *in vitro* mutagenesis, to dissect the catalytic and structure-function relationship thereof and permit a better design and identification of the resulting proteins.

15   The term "nucleic acid construct" refers to a strand of nucleic acid composed of nucleic acid sequences which do not normally co-exist. A nucleic acid construct is generally prepared for a specific purpose, and thus, incorporates all the components necessary to achieve that purpose. For example, a nucleic acid construct encoding a specific protein can be prepared which includes the components necessary for the

20   expression of that protein under certain conditions. Vector DNA incorporating exogenous DNA, as discussed below, is an example of a nucleic acid construct.

A host cell or indicator cell has been "transfected" by exogenous or heterologous DNA or RNA (e.g. a DNA construct) when such RNA or DNA has been introduced into the cell. The transfecting RNA or DNA may or may not be integrated

25   (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, the transfecting/transforming DNA may be maintained on an episomal element such as a plasmid. On the other hand, in eukaryotes, a stably transfected cell is one in which the transfecting DNA becomes integrated with the genomic DNA in chromosomes and is inherited by daughter cells through chromosome replication.

30   Furthermore, in eukaryotes, a stably transfected cell may be one in which the transfecting RNA is maintained as an episomal element such as a replicon. This

-10-

stability is demonstrated by the ability of the cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA. Transfection methods are well known in the art as described in Sambrook *et al.*, 1989 and Ausubel *et al.*, 1994.

5   The term "replicon" as used herein means an RNA molecule that can encode one or more protein molecules and replicates through a complementary RNA strand intermediate.

In order to readily evaluate expression of a gene of interest, a cell can be transfected with a gene that encodes a detectable marker or "reporter" protein along with the
10   gene of interest such that expression of the reporter protein will be indicative of expression of the gene of interest. The term "reporter gene" refers to a nucleotide sequence encoding such a "reporter protein". Examples of commonly used reporter genes include secreted alkaline phosphatase (SEAP), neomycin, luciferase, chloramphenicol amino transferase (CAT), P-galactosidase and green fluorescent
15   protein (GFP).

The term "selectable marker" as used herein means a gene that, when expressed, renders the cell resistant to a selection agent such as an antibiotic (also referred to as selective pressure).

The term "selective pressure'" or "selection agent" as used herein interchangeably
20   mean a molecule or compound that, when presented to cells that do not express the selectable marker, will induce cell death. For example, such selection agents can include antibiotics such as: G418, hygromycin, zeomycin or puromycin.

The designation "variant" denotes in the context of this invention an NS3 protease that exhibits inhibitor-resistance as set out above. A variant may be from the same or
25   different species and may be a natural variant or a synthetically derived variant. A variant NS3 protease in accordance with the present invention may incorporate one or more amino acid substitutions, deletions, or additions provided that inhibitor-resistance is conserved. Variations in the nucleotide sequence encoding the present mutant NS3 protease are also encompassed, and these may also include substitutions,
30   deletions, or additions of one or more nucleotides within the sequence provided that

-11-

the inhibitor-resistance of the protease encoded by the nucleotide sequence is conserved. Variant, derivative and fragment molecules in accordance with the present invention, including both protein and nucleic acid molecules, can be obtained using methods well-known in the art including techniques of isolation/purification,

5   chemical synthesis and recombinant DNA technology.

The term "vector" refers to a nucleic acid compound used for introducing exogenous nucleic acids into host cells. A vector comprises a nucleotide sequence which can encode one or more protein molecules. Plasmids, cosmids, viruses and bacteriophages, in the natural state or which have undergone recombinant

10   engineering, are examples of commonly used vectors, into which an exogenous genetic sequence or element (either DNA or RNA) may be inserted so as to bring about the replication of the exogenous sequence or element.

Preferred Embodiments

15   Method of selecting and isolating mutant inhibitor-resistant NS3 protease

In a first embodiment of the present invention, there is provided a method of preparing a mutant inhibitor-resistant HCV NS3 protease.

The method comprises the steps of preparing a nucleic acid construct encoding a selectable marker and native HCV NS3 protease wherein the replication and

20   expression of the selectable marker is dependent on HCV NS3 protease activity. An example is the HCV replicon. Host cells are transfected with this construct and incubated in the presence of an NS3 inhibitor under selective pressure under conditions suitable for selection of successfully transfected cells. Examples of selectable markers are identified above. The appropriate selectable marker will.

25   confer on successfully transfected cells a growth advantage under the growth conditions provided to the cells. Colonies resulting from the incubation under these conditions incorporate mutant inhibitor-resistant HCV NS3 protease.

Methods for lysing the cells and isolating the mutant nucleic acid, or ultimately isolating the mutant protease, are well known within the skill of the art, or as

30   described in the following examples.

-12-

The nature of the mutations which render the NS3 mutant proteases resistant to the selected inhibitor can readily be identified using standard sequencing techniques which are well-known to those of skill in the art.

Following the first identification of the relevant mutants, validation by further

5   production of similar mutant nucleic acids or proteases can be obtained by site-directed mutagenesis by techniques well-known by the person of skill in the art, or as is described in the following examples.

Mutant proteases

10   The present invention provides, in a second embodiment, a novel inhibitor-resistant HCV NS3 mutant protease.

While not bound by any particular theory, in one aspect of this embodiment the mutations to the native NS3 protease, result in a change, perhaps conformational or steric, that prevents, or at least reduces, protease-inhibitor binding such that protease

15   activity is substantially retained. Particularly, mutations in and around amino acids 1-180 of the protease can be mutated such that inhibitor-binding is decreased. More particularly, mutations in and around the active site or close to the inhibitor binding-site can be mutated such that inhibitor-binding is decreased.

In one aspect of this embodiment, the inhibitor-resistant protease is mutated at least

20   one of the amino acid position corresponding to amino acids 155, 156 or 168 of the native HCV NS3 protease sequence. The native amino acid at position 155 is arginine (Arg155 or R155), position 156 is alanine (Ala156 or A156) and position 168 is most often in genotype 1 an aspartic acid (Asp168 or D168), though glutamic acid (Glu or E) is also found in some genotype 1 viruses, or glutamine (Gln or Q) is

25   found in genotype 3 viruses.   An example of the native amino acid sequence of HCV-1b NS3 protease domain is shown in **SEQ ID No: 2**.

In accordance with a preferred aspect of the present invention, the inhibitor-resistant NS3 protease has at least one of the amino acid at positions 155, 156 and 168 that is replaced with an amino acid that is not naturally occurring in the HCV NS3 protease

30   at these positions.

-13-

The native amino acid at position 155 is arginine (Arg^{155} or R^{155}). In accordance with a preferred aspect of the present invention, the inhibitor-resistant NS3 protease in which the arginine at position 155 is replaced with a non-basic amino acid. Replacement of Arg^{155} generally with non basic amino acids such as glutamine (Q) or tryptophan (W) results in an inhibitor-resistant NS3 protease.

In accordance with a preferred aspect of the present invention, the inhibitor-resistant NS3 protease has the alanine at position 156 that is replaced with any other amino acid. In another aspect of this embodiment, the inhibitor-resistant protease is mutated at Ala156 generally with uncharged amino acids such as glycine (G), threonine (T) or valine (V), resulting in an inhibitor-resistant NS3 protease. Alternatively, the inhibitor-resistant protease is mutated at Ala156 generally with uncharged amino acids having a slightly larger side chain such as threonine (T) or valine (V), resulting in an inhibitor-resistant NS3 protease.

In another aspect of this embodiment, the inhibitor-resistant protease is mutated at amino acid position 168. The native amino acid at position 168 is aspartic acid (Asp^{168} or D^{168}) glutamic acid (Glu or E) or glutamine (Gln or Q). In accordance with a preferred aspect of the present invention, the inhibitor-resistant NS3 protease has the aspartic acid/glutamic acid/glutamine at position 168 that is replaced with any other amino acid. Replacement of Asp^{168} generally with amino acids such as glycine (G), alanine (A), asparagine (N), histidine (H) or valine (V) results in an inhibitor-resistant NS3 protease. Preferably, the inhibitor-resistant NS3 protease has the aspartic acid/glutamic acid/glutamine at position 168 that is replaced with an uncharged amino acids with small side chains such as glycine (G), alanine (A), asparagine (N) or valine (V) results in an inhibitor-resistant NS3 protease. More preferably, the Asp/Glu/Gln168 is mutated to alanine (A) or valine (V). Alternatively, in a further preferred embodiment, the native Asp/Glu/Gln^{168} of the NS3 protease is replaced with glycine (G), asparagine (N) or histidine (H).

In a further preferred aspect of this embodiment, the native Arg^{155} of the NS3 protease is replaced with glutamic acid.

In a preferred aspect of this embodiment, the native Ala^{156} of the NS3 protease is

-14-

replaced with valine.

In a further preferred aspect of this embodiment, the native Asp^{168} of the NS3 protease is replaced with valine.

In a preferred aspect of this embodiment, at least one of native Ala^{156} and Asp^{168} of

5   the NS3 protease is replaced with valine.

In a further preferred aspect of this embodiment, both of native Ala^{156} and Asp^{168} of the NS3 protease are replaced with valine.

Another aspect of the present invention covers a NS3 protease domain comprising a sequence having 90% identity to the sequence shown in **SEQ ID No.2** wherein the

10   amino acid sequence is mutated as defined above.

Alternatively, in a further preferred aspect of this embodiment, any one of the mutations presented in Tables 1, 2, 3 or 4 can lead to an inhibitor-resistant mutant HCV NS3 protease.

15   Recombinant nucleic acid molecules

In a third embodiment, the invention encompasses recombinant nucleic acid molecules, including both DNA and RNA molecules that encode mutant HCV inhibitor-resistant NS3 proteases as defined above. An example of the nucleotide sequence encoding a native HCV NS3 protease domain is set out in **SEQ ID No: 1**.

20   Modifications in this sequence which encode mutant inhibitor-resistant NS3 proteases as determined using methods known to those of skill in the art, such as the cell-based assays described in the specific examples herein, are encompassed, including variants, derivatives and fragments thereof. These sequence modifications may also, of course, incorporate modifications that result from the degeneracy of the

25   nucleic acid code.

In one aspect of the present invention, the nucleic acid molecule encodes a mutant HCV inhibitor-resistant NS3 protease which is modified at at least one of the amino acid as defined above.

Nucleic Acid Constructs, Vectors, Replicon and Viruses

In a fourth embodiment, the invention encompasses nucleic acid constructs, vectors, replicons and viruses comprising a nucleic acid molecule encoding a mutant HCV inhibitor-resistant NS3 protease as described above.

5   In one aspect of this embodiment, nucleic acid constructs encoding inhibitor-resistant NS3 protease are prepared in which nucleic acid encoding the mutant protease is linked to nucleic acid encoding a selectable marker.

In a particular aspect of this embodiment, the mutant HCV inhibitor-resistant NS3 protease-encoding nucleic acid is incorporated "expressibly" into a construct or

10   vector. In order to achieve expression, the protease-encoding nucleic acid is linked to elements within the construct or vector which are appropriate to allow for expression of the protease-encoding nucleic acid on transfection, stably or transiently, into a host cell. Expression constructs and vectors are well-known to those of skill in the art as described above.

15

Transfected host cells

In a fifth embodiment, host cells transfected with an expression vector or a replicon comprising a nucleic acid molecule encoding a mutant HCV inhibitor-resistant NS3 protease are provided, as well as cell lines derived from such host cells.

20   In one aspect of the preferred embodiment, there is provided appropriate expression systems and prokaryotic or eukaryotic cells for expression of the mutant HCV NS3 protease.

Preferred aspects of this embodiment, provide expression systems in *E. coli*, baculovirus, yeast as well as mammalian cells such as in Huh-7 cells.

25   In a preferred aspect of this embodiment, there is provided a mammalian host cell transfected with a vector or a replicon, or infected with a virus, comprising a nucleic acid molecule encoding a mutant inhibitor-resistant NS3 protease, either stably or transiently. Non-limiting examples of appropriate mammalian host cells and cell lines include primary hepatocytes, liver cell lines, fibroblasts, lymphocytes, and

-16-

kidney cell lines.

In a further preferred aspect, a human host cell line is provided that is transfected with a mutant inhibitor-resistant NS3 protease-encoding nucleic acid. In a more preferred aspect, the transfected human host cell line is selected from a liver or kidney cell line. Examples of preferred host cells include human embryonic kidney cells of the 293 lineage (ATCC CRL 1573), human carcinoma cells including those of the HeLa lineage (ATCC CCL 2), and neuroblastoma cells of the lines IMR-32 (ATCC CCL 127), SK-N-MC (ATCC HTB 10) and SK-N-SH (ATCC HTB 11), Huh-7 cells, WRL68 cells, HepG2 cells and Chang cells.

Method for evaluating protease activity

In a sixth embodiment of the present invention, there is provided a method of evaluating NS3 protease activity of a mutant HCV NS3 protease in accordance with the present invention.

The method comprises the steps of incubating host cells transfected with nucleic acid encoding an inhibitor-resistant NS3 protease under conditions which cause the protease to be expressed; and measuring the replication of the nucleic acid, wherein the level of replication is proportional to the activity of the expressed protease. As one of skill in the art will appreciate, the level of replication as well as the protease activity can be measured (directly or indirectly) using standard assays established for this purpose, such as the assays described in detail in the specific examples that follow.

Methods for evaluating protease activity include but are not limited to, replicon assays, surrogate cell based assays, or purified NS3 protease *in vitro* enzymatic assays.

Method for identifying inhibitors

In a seventh embodiment of the present invention, there is provided a method of screening candidate second generation inhibitor compounds for the ability to inhibit

-17-

the activity of mutant HCV NS3 protease in accordance with the present invention.

In an eighth embodiment of the present invention, there is provided a method of screening candidate second generation inhibitor compounds for the ability to inhibit the activity of mutant HCV NS3 protease in cell-free assay in accordance with the

5   present invention.

In one aspect, the method of identifying potential second generation inhibitor compounds of HCV NS3 protease comprises incubating an isolated inhibitor-resistant NS3 protease mutant as defined above in the presence or absence of a candidate second generation inhibitor compound; and measuring the protease

10   activity of the inhibitor-resistant NS3 protease in the presence and absence of the candidate second generation inhibitor compound. A decrease in activity in the presence of a candidate second generation inhibitor compound indicates that the compound inhibits the inhibitor-resistant NS3 protease.

In another aspect, the method of identifying potential second generation inhibitor

15   compounds of an HCV NS3 protease comprises incubating host cells transfected with nucleic acid encoding an inhibitor-resistant NS3 protease in the presence of a candidate second generation inhibitor compound under conditions which cause expression thereof; incubating host cells transfected with nucleic acid encoding an inhibitor-resistant NS3 protease in the absence of a candidate second generation

20   inhibitor compound under conditions which cause expression thereof; and then measuring the replication of the nucleic acid, wherein the level of replication of the nucleic acid is proportional to the protease activity in each case. A decrease in protease activity in the presence of a candidate second generation inhibitor compound indicates that the candidate compound inhibits the protease.

25   The cell-based assays and methods of the present invention are conducted under conditions for mammalian cell growth that are well known to a person skilled in the art, i.e. physiological pH, salt concentrations using buffers such as PBS, temperatures between 30° and 42°, appropriate cell culture media and providing sufficient time for cell growth. More specifically, the transfected host cells are incubated for a

30   sufficient time to allow for expression of the mutant NS3 inhibitor-resistant protease,

-18-

for example, an incubation period of at least 1 hour, but more preferably, an incubation period of from 10 hours to about 24 hours.

The assays and methods of the present invention are conducted under conditions for measuring NS3 protease activity that are well known to a person skilled in the art,

5   i.e. physiological pH, salt concentrations using buffers such as Tris, HEPES, temperatures between 15° and 37° (preferably at room temperature), appropriate incubation and detection techniques.

Preferred aspects of embodiments of the present invention are described in the following specific examples which are not to be construed as limiting.

10

EXAMPLES

Example 1 - Identification of HCV NS3 Mutants Resistant to a Macrocyclic Peptide HCV Inhibitor.

The method described by Lohmann et al. (1999, Science 285:110) was used to mimic

15   the replication of subgenomic HCV RNA in a system that is dependent on the function of HCV non-structural proteins and enzymes. This HCV RNA replicon system incorporates two cistrons: one encoding the HCV non-structural region and the second encoding a selectable neomycin resistant marker (i.e. gene encoding neomycin phosphotransferase). As one of skill in the art will appreciate, the second

20   cistron may encode any marker suitable for selection purposes. Cell lines harboring such bicistronic, subgenomic HCV RNA, such as the S22.3 cell line, are described by Kukolj and Pause (see WO 02/052015, the contents of which are hereby incorporated by reference). These cell lines are useful in evaluating the efficacy and potency of potential anti-HCV therapeutics that inhibit one or more of the HCV non-

25   structural proteins.

The present method by which HCV RNA replication was established in cell culture is summarized in Figure 1. As shown, Huh-7 cells (8×10^6 cells) were transfected with the bicistronic subgenomic HCV RNA using transfection methods well-established in the art. Transfected Huh-7 cells were selected by growth in neomycin-

30   containing media (0.5 mg/ml neomycin). Specifically, transfected cells replicate the

-19-

RNA that produces neomycin phosphotransferase thereby rendering the cells resistant to the cytotoxic effect of neomycin. Mutated HCV replicons as described in WO 02/052015 can also be used in accordance with the present method to increase RNA replication and efficiency of replicon establishment.

5   In short, the selection of S22.3 cell lines resistant to NS3 protease inhibitors was as follows: S22.3 cells were trypsinized and resuspended in fresh media (DMEM, 10% fetal calf serum) with 1 mg/ml G418 antibiotic. 150,000 cells were plated into one well of a 6-well plate. The following day (t= day 0), fresh medium containing the NS3 protease inhibitor at a pre-determined concentration and 1 mg/ml G418 was

10   added to the well. After cells reached confluency on day 3, the S22.3 cells were trypsinized and passed into a 10 cm plate. Medium (containing inhibitor and 1mg/ml G418) was changed on day 6 and day 10. By day 11, cell death was evident as, the majority of cells had lost resistance to G418. At day 14 fresh medium was changed which now contained a pre-determined concentration of NS3 protease inhibitor with

15   0.5 mg/ml G418. Medium with inhibitor and 0.5 mg/ml G418 was replenished at day 17 and day 20. The cells that formed colonies by day 21 were picked for expansion into 48-well plates, and the colonies were counted by fixing and staining the cells with crystal violet. Resistant cell lines were propagated from 48-well to 24-well to 6-well and beyond. The total cellular RNA that also contained the HCV subgenomic

20   replicon RNA was isolated from 1,000,000 cells (or more) by Qiagen RNeasy® cartridge and protocol. HCV sequence was amplified by RT-PCR and the NS3 region was sequenced with NS3 specific primers.

Specifically, neomycin-resistant cell colonies were then exposed to a known NS3 protease inhibitor, specifically, a macrocyclic peptide inhibitor:

25   **inhibitor A**= cyclopropa[e]pyrrolo [1,2-a][1,4]diazacyclopentadecine-14a(5H)-carboxylic acid, 6-[[(cyclopentyloxy) carbonyl]amino]-1,2,3,6,7,8,9,10,11,13a, 14,15,16,16a-tetradecahydro-2-[[7-methoxy-2-[2-[(1-methylethyl)amino]-4-thiazolyl]-4-quinolinyl]oxy]-5,16-dioxo-, (2R,6S,12Z,13aS,14aR,16aS) ,

which is described as compound #822 in WO 00/59929, the contents of which is

30   hereby incorporated by reference. The colonies were grown in the presence of

-20-

increasing concentrations of this inhibitor (EC$_{50}$ of 2 nM) ranging from 2nM to 2000 nM.

Colonies emerging in the presence of low (6xEC$_{50}$ or 12 nM) and high (1000xEC$_{50}$ or 2000 nM) concentrations of **inhibitor A** were expanded and the replicon was RNA sequenced over the NS3/4A region using standard sequencing techniques. Mutations detected in the NS3 protease domain at both high and low inhibitor concentrations are summarized below in Table 1.

Amino acids are referred by 1-letter code, the native HCV NS3 protease amino acid preceding its position number in the protease and the mutant amino acid following the position number, e.g. D168G indicates the native aspartic acid at position 168 is replaced by glycine in the mutant protease.

TABLE 1

Low Inhibitor [ ] (12 nM)		High Inhibitor [ ] (2000nM)	
nucl change	AA change	nucl change	AA change
A503G	D168G	G466A - A503G	A156T D168A
A211G - G466A	I71V A156T	C467T	A156V
A257G - A503G	Q86R D168G	C467T	A156V
A503G	D168G	C467T	A156V
C467T	A156V	C467T	A156V
A503G	D168G	C265T - C467T	P89S A156V
G464A	R155Q	C265T - C467T	P89S A156V
A503T	D168V	A503T	D168V
A122G	Q41R	C467T	A156V
C467T	A156V	C266T - C467T	P89L A156V
A503G	D168G	C467T	A156V
G502R	D168N/D	C467T	A156V
G502A - A532T	D168N T178S	A503T	D168V

Low Inhibitor [ ] (12 nM)		High Inhibitor [ ] (2000nM)	
A239G - G331T	Q80R A111S	A503T	D168V
G502C	D168H	A503T	D168V
C467T	A156V	C467T	A156V
C467T	A156V	G466A	A156T
A257G - A503G	Q86R D168G	C467T	A156V
C315T	D168E	C467T	A156V
C467T	A156V	C467T	A156V
G526A	E176K		

The results show that a variety of amino acid mutations occurred in the proximity of the active site (see black amino acids in Figure 3), particularly in colonies isolated at low inhibitor concentration. The mutations, alanine to valine or threonine at position

5   156 (A156V/Y) and aspartic acid to valine/alanine at position 168 (D168V/A) were consistently observed in colonies isolated at high inhibitor concentration. In particular, the mutation, A156V, was detected in 70% of the sequenced colonies grown at high inhibitor concentration, while the mutation, D168V, was detected in 20% of the sequenced colonies grown at high inhibitor concentration.

10

Example 2 – Identification of HCV NS3 Mutants Resistant to NS3 Inhibitor B.

The method described above in Example 1 was used to identify NS3 protease mutants that occurred by growth in the presence of NS3 protease inhibitor:

inhibitor B= cyclopropane carboxylic acid, N-[(cyclopentyloxy)carbonyl]-3-methyl-

15   L- valyl-(4R)-4-[[2-[2-(acetylamino)-4-thiazolyl]-7-methoxy-4-quinolinyl]oxy]-L-prolyl-1-amino-2-ethenyl-, (1R,2S)-, which has an EC_{50} of 40 nM.

Colonies emerging in the presence of low (3xEC_{50} or 120 nM) and high (50xEC_{50} or 2000 nM) concentrations of inhibitor were expanded and the replicon RNA was sequenced over the NS3/4A region using standard sequencing techniques. Mutations

20   detected in the NS3 protease domain at both high and low inhibitor concentrations

-22-

are summarized below in Table 2.

TABLE 2

Low Inhibitor [ ] (120 nM)		High Inhibitor [ ] (2000 nM)	
nucl change	AA change	nucl change	AA change
C467G	A156G	C467T	A156V
G115A - C430T - A503T	A39T L144F D168V	C467T	A156V
A503G	D168G	C467T	A156V
C266T - G464A	P89L R155Q	C467T	A156V
C467T	A156V	A503T	D168V
G77A - A503G - A535G	R26K D168G M179V		
C467T - G537A	A156V M179I		
A122G - A503G	Q41R D168G		
C366A	S122R		
C265T - C366A	P89S S122R		
A211G - G502A	I71V D168N		
G502A	D168N		
A503C	D168A		
C266T - G464A	P89L R155Q		

Again, the results show that a variety of amino acid mutations occurred in the
proximity of the inhibitor binding pocket, particularly in colonies isolated at low
inhibitor concentration, and the individual mutations, A156V/G, and D168V/N/A/G
were consistently observed in colonies isolated at low and high inhibitor
concentration. The mutation, A156V, was detected in 80% of the sequenced
colonies grown at high inhibitor concentration, while the mutation D168V was
detected in 20% of the sequenced colonies grown at high inhibitor concentration.

-23-

Example 3 - Identification of HCV NS3 Mutants Resistant to Another NS3 Inhibitor.

The method described above in Example 1 was used in conjunction with NS3

5   protease inhibitor:

inhibitor C= Cyclopropa[e]pyrrolo[1,2-a][1,4]-diazacyclopentadecine-14a(5H)-carboxylic acid, 2-[[2-[2-(acetylamino)-4-thiazolyl]-7-methoxy-4-quinolinyl]oxy]-6-[[(1,1-dimethylethoxy)carbonyl]amino]-1,2,3,6,7,8,9,10,11,13a,14,15,16,16a-tetradecahydro-5,16-dioxo-, (2R,6S,12Z,13aS,14aR,16aS)-,

10   which is also described as compound #702 in WO 00/59929 (incorporated herein by reference) which has an EC_{50} of 5 nM.

Colonies emerging in the presence of low ($6xEC_{50}$ or 30 nM), intermediate ($20xEC_{50}$, 100nM) and high ($100xEC_{50}$, 500nM and $200xEC_{50}$, 1000 nM) concentrations of inhibitor were expanded and the replicon RNA was sequenced over

15   the NS3/4A region using standard sequencing techniques. Mutations detected in the NS3 protease domain at different concentrations are summarized below in Table 3.

TABLE 3

Low Inhibitor [ ] (30 nM)		Intermediate [ ] (100nM)		High Inhibitor [ ] (500nM)		High Inhibitor [ ] (1000nM)	
nucl change	AA change	nucl change	AA change	AA change	AA change	nucl change	AA change
G466A	A156T	G466A	A156T	A84G A504T	Q28R D168V	A503T/C	D168V
C449T C467T	A150V A156V	A504C	D168A	A84G A504T	Q28R D168V	A503T	D168V
G59A C343T C430T A503G	S20N P115S L144F D168G	A504T	D168V	C467T	A156V	G466A	A156T
G466A	A156T	G466A	A156T	A504T	D168V	C467T	A156V
A239G	Q80R	G466A G472C	A156T V158L	A504T T536C	D168V M179Y	A503T	D168V
G466A	A156T					C467T	A156V
C463T	R155W					G302A A503C	S101N D168A
A239G	Q80R						
A503T	D168V						
C467T	A156V						

The results show that a variety of amino acid mutations occurred in the proximity of the active site, and the mutations A156V/T, and D168V/A/G were consistently observed.

Example 4- Identification of HCV NS3 mutants resistant to a macrocyclic peptide HCV inhibitor in another cell line harboring a bicistronic subgenomic HCV RNA.

Cell line R3 was derived from S22.3 and contains bicistronic HCV replicon with mutations that confer adaptivity to replicate in Huh7 cells (WO 02/052015 incorporated herein by reference). **Inhibitor A** described above in Example 1 was used to identify NS3 protease mutants in R3 cell lines.

-25-

Colonies emerging in the presence of 7.5 nM and 700 nM of **inhibitor A** were expanded and the replicon RNA was sequenced over the NS3/4A region using standard sequencing techniques. Mutations detected in the NS3 protease domain at both concentrations are summarized in Table 4.

5

TABLE 4

Inhibitor [7.5 nM]		Inhibitor [700 nM]	
nucl. change	AA change	nucl. change	AA change
A503G	D168G	A503T	D168V
A503T	D168V	G466A	A156T
A503C	D168A	A503C/T	D168A,V
G464A	R155Q		
G502A,G464A	D168N,R155Q		
C467T	A156V		

The results show that a selected number of amino acid mutations occurred in the proximity of the active site. The individual mutations, aspartic acid to either valine (D168V), or alanine (D168A) or glycine (D168G) were observed more frequently at

10 the low concentration of inhibitor while D168V was more frequently observed at high concentration of inhibitor.

EXAMPLE 5 – DETERMINATION OF INHIBITOR EC$_{50}$ USING NS3 MUTANT REPLICON CELL LINES

15 Specific isogenic HCV subgenomic replicon cDNAs individually containing only one of the inhibitor-resistant mutations, i.e. (in this example: A156T; or A156V; or D168V; or D168G) were cloned and corresponding *in vitro* transcribed RNA of these modified bi-cistronic replicons were generated. The mutant-encoding RNAs were used to construct mutant-containing replicon cell lines in naïve Huh-7 cells as

20 described previously for other HCV replicons. Each of these individual replicon cell

-26-

lines were then used to confirm the decrease in inhibition exhibited by NS3 protease **inhibitor A** on the NS3 mutants. They were also used to determine the level of inhibition of the mutant inhibitor-resistant cell lines in the presence of an unrelated HCV NS5B polymerase inhibitor or alpha-interferon. Using the assay described in

5   WO 03/010141 (Example 48) the EC_{50} of each inhibitor was determined using the mutant cell lines. The results of these experiments are summarized below in Table 5.

TABLE 5

Inhibitor					
	WT	**A156T**	**A156V**	**D168V**	**D168G**
A	0.0009	0.547	0.324	0.735	0.023
polymerase inhibitor	1.39	1.34	1.19	1.38	1.13
IFN-α	0.23	0.21	0.23	0.20	0.18

Note that each of these four mutants only shift the potency of the related NS3 protease inhibitors and have no effect on the EC_{50} of an unrelated HCV NS5B

10   polymerase inhibitor or interferon-alpha. The D168V and A156T mutants are most detrimental to this class of NS3 protease inhibitors, shifting the cell culture EC_{50} potency up to 700-fold.

EXAMPLE 6 – IN VITRO ENZYME ASSAYS AND DETERMINATION OF Ki

15   DNA encoding the NS3-NS4A coding region of HCV genotype 1b, that includes a 28-residue N-terminal sequence containing a hexahistidine tag and a TEV protease cleavage (**Pause, A. et al. (2003)** *J. Biol. Chem.* 278: 20374-20380) was amplified by PCR and then subcloned into the pET11a bacterial expression vector. Amino acid substitutions A156T and D168V were introduced into the NS3-NS4A coding region

20   using standard site-directed mutagenesis procedures.

Alternatively, the NS3 protease (1-180) coding region for wild-type with the sequence ASKKKK at the C-terminus was cloned into a bacterial expression vector and amino acid substitutions A156T, D168V and R155Q were introduced as

described above.

The NS3 protease domain (harboring A156T or D168V or R155Q mutations) were expressed in *E. coli* and purified as previously described (**LaPlante, S.R. et al (1999)** *J. Biol. Chem.* **274**: 18618-18624). The NS3-NS4A proteins with either

5   A156T or D168V mutation were also expressed in *E. coli* and the cell paste was resuspended in 5 mL of lysis buffer (50 mM sodium phosphate, pH 7.5, 20% glycerol, 1 mM EDTA, 0.5% Triton X-100, 0.5 M NaCl) per gram of cells, treated with DNase I, then clarified by a 30-min centrifugation at 150,000×*g*; the supernatant was diluted 2-fold in 50 mM sodium phosphate, pH 7.5, 0.5 M NaCl and imidazole

10   was added to a final concentration of 25 mM. The NS3-NS4A complex solution was purified by successive steps on a Hi-Trap Ni^{+2}-chelating column, and poly(U)-Sepharose affinity column previously equilibrated in 50 mM sodium phosphate, pH 7.0, 10% glycerol, 0.2 M NaCl, 0.05% n-dodecyl-β-D-maltoside, 10 mM β-mercaptoethanol. The enzyme was eluted in the same buffer containing 2 M NaCl.

15   The isolated enzymes were stored at -80°C.

K_i determination - The inhibition constant (K_i) was determined using the depsipeptide fluorogenic substrate anthranilyl-DDIVPAbu[C(O)-O]AMY(3-NO_2)TW-OH. The cleavage reaction was continuously monitored at 23°C on a

20   BMG POLARstar® Galaxy fluorometer, equipped with excitation and emission filters of 320 and 405 nm respectively. The NS3 protease wild type and mutant (5-20 nM) were assayed in 50 mM Tris-HCl, pH 7.5, 30% glycerol, 1 mg/mL BSA, 1 mM TCEP in the presence of a 1000-fold molar excess of the NS4A$_{peptide}$. A 15-min pre-incubation was carried out to allow the formation of the NS3 protease-

25   NS4A$_{peptide}$ complex. The NS3-NS4A proteins (5-20 nM) were assayed in 50 mM Tris-HCl, pH 8.0, 0.25 M sodium citrate, 0.01% n-dodecyl-β-D-maltoside, 1 mM TCEP. The initial velocity of the inhibited reaction was determined at several inhibitor concentrations (corresponding to a 25-75% inhibition range) while varying the substrate concentration (typically from 0.1 to 5.0 K_m), assuming Michaelis-

30   Menten kinetics. The reaction was initiated by enzyme addition. K_i calculations were performed by non-linear regression analysis of the initial velocity data using the

GraFit software (version 3.0, Erithacus® Software Ltd., Staines, UK). Some K_i values were calculated from the IC_{50} (50% effective concentration) based on the following equation for competitive inhibition: $IC_{50} = 0.5E_t + K_i (1 + S/K_m)$. IC_{50}'s were obtained from a non-linear curve fit using the Hill model applied to the %
5   inhibition-concentration data and calculated through the use of SAS (Statistical Software System, SAS Institute Inc., Cary, N.C.).
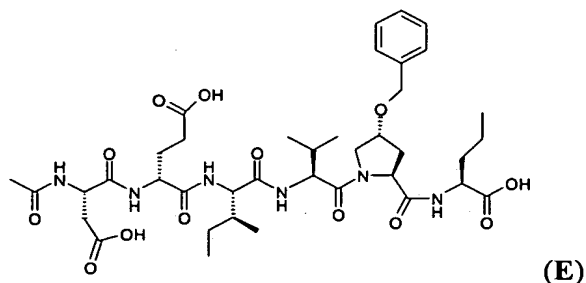
The results shown in Tables 6 and 7 summarize *in vitro* sensitivity of some NS3 protease inhibitors (as observed K_i values) to the NS3 protease WT versus the
10   inhibitor-resistant mutant in the form of either the full length NS3/NS4A protein (Table 6) or the NS3-NS4A peptide complex (Table 7). Note that the shift in K_i values for **inhibitor A**, (i.e. the K_i obtained with the mutant enzymes relative to the wild type enzyme) resembles the shift observed in EC_{50} with this inhibitor in the cell culture assay (Table 5).

15

TABLE 6

Inhibitor	Ki (nM)		
	WT	A156T	D168V
E	1600	n.d.	8100
F	0.047	29	12
A	1.6	870*	520*

*calculated K_i from IC_{50}

Compound **E** has the following structure:

(E)

-29-

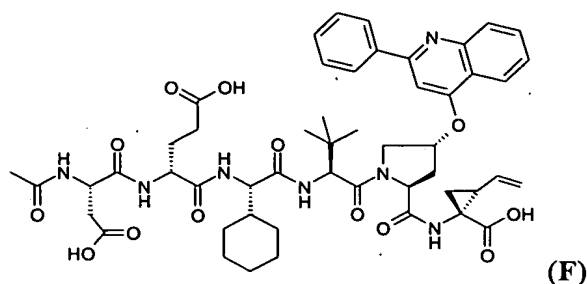Compound **F** has the following structure:

(F)

TABLE 7

Inhibitor	K_i (nM)			
	WT	A156T	D168V	R155Q
A	5.6	800*	970*	220*

*calculated K_i from IC_{50}

Conclusion

A method for the selection of HCV sub-genomic RNAs that replicate in the presence of various inhibitors of HCV NS3 protease is described in the foregoing examples. This method for isolating HCV replicons that are resistant to NS3 protease inhibitors, permits the identification and prediction of nucleotide and amino acid substitutions that may confer a growth advantage to the HCV virus in the presence of these inhibitors, related inhibitors and, indeed, any other known or future HCV inhibitor particularly , NS3 protease inhibitors that inhibit the enzyme by the same mechanism or binding to the same region/pocket.

The *a priori* knowledge of mutations which render HCV resistant to NS3 protease inhibitors provides the basis for identifying inhibitors that will be effective against such resistant strains, for example, using high-throughput *in vitro* assays that reconstitute activities of the resistant HCV enzyme or protein. The mutant HCV NS3 proteases (harboring any one of the mutations described herein) that retain activity but are not inhibited by "first-generation" inhibitors are valuable tools in identifying

second generation inhibitors that may be required in multi-drug combination therapy for the treatment of HCV infection.

References

5   Ausubel *et al.*, 1994, Current Protocols in Molecular Biology, Wiley, New York

Gale Jr. *et al.*, 1997, Virology 230, 217

Kim JL, *et al.* 1996, Cell;87:343-355

Kukolj and Pause, WO 02/052015

Lohmann *et al.*, 1999, Science 285:110

10   Love, R.A. *et al.*, 1996, Cell, 87, 331-342

Reed *et al.*, 1997, J. Virol. 71, 7187

Remington, 1995, The Science and Practice of Pharmacy

Sambrook *et al.*, 2000, Molecular Cloning – A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press.

15   Trozzi *et al.*, 2003 J. Virol, 77, 3669-3679 (corresponds to Migliaccio *et al.*, 2002, *Selection and Characterization of HCV Replicon Mutants; Resistant to Antiviral Agents.*, Abst. 9th International Meeting of HCV and Related Viruses, Jul 7-11, La Jolla, California).